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Effect of dietary sodium modulation on pig adrenal steroidogenesis and transcriptome profiles

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Running title: Pig adrenal response to sodium restriction

1 **Abstract**

2 Primary aldosteronism is a frequent form of endocrine hypertension caused by aldosterone
3 overproduction from the adrenal cortex. Regulation of aldosterone biosynthesis has been studied
4 in rodents despite differences in adrenal physiology with humans. We therefore investigated pig
5 adrenal steroidogenesis, morphology, and transcriptome profiles of the zona glomerulosa (zG) and
6 zona fasciculata (zF) in response to activation of the renin-angiotensin-aldosterone system by
7 dietary sodium restriction. Six-week-old pigs were fed a low or high sodium diet for 14 days (3 pigs
8 per group, 0.4g sodium/kg feed *versus* 6.8g sodium/kg). Plasma aldosterone concentrations
9 displayed a 43-fold increase ($p=0.011$) after 14-days of sodium restriction (day 14 *versus* day 0).
10 Low dietary sodium caused a 2-fold increase in thickness of the zG ($p<0.001$) and an almost 3-fold
11 upregulation of *CYP11B* (cytochrome P450 11B1) ($p<0.05$) compared with high dietary sodium.
12 Strong immunostaining of the KCNJ5 potassium channel, which is frequently mutated in primary
13 aldosteronism, was demonstrated in the zG. mRNA-seq transcriptome analysis identified
14 significantly altered expression of genes modulated by the renin-angiotensin-aldosterone system
15 in the zG ($n= 1,172$) and zF ($n= 280$). These genes included many with a known role in the
16 regulation of aldosterone synthesis and adrenal function. The most highly enriched biological
17 pathways in the zG were related to cholesterol biosynthesis, steroid metabolism, cell cycle and
18 potassium channels. This study provides mechanistic insights into the physiology and
19 pathophysiology of aldosterone production in a species closely related to humans and shows the
20 suitability of pigs as a translational animal model for human adrenal steroidogenesis.

Key words: aldosterone; cortisol; hyperaldosteronism; adrenal cortex; sodium restriction;
steroidogenesis; hypertension

21 **Introduction**

22 The mineralocorticoid hormone aldosterone is synthesized in zona glomerulosa (zG) cells of the
23 adrenal cortex and stimulates sodium reabsorption in epithelial cells of the kidney distal tubule
24 and colon for the maintenance of blood volume and blood pressure. The main physiological
25 regulators of aldosterone production are the renin-angiotensin-aldosterone system (RAAS) and
26 circulating potassium; although other factors are likely also involved.¹⁻³ Sodium depletion activates
27 the RAAS and causes expansion of the zG layer and an increase in aldosterone secretion.^{4,5}
28 Manipulation of dietary sodium in rats has been used as an approach to study the effects of RAAS
29 activation on zG gene expression to identify genes that function in the regulation of aldosterone
30 production.⁵⁻⁷

31
32 Humans and commonly used experimental surrogates display distinct differences in adrenal
33 physiology. For example, the *Cyp17a1* gene (encoding 17 α -hydroxylase and 17,20-lyase) is not
34 expressed in the adrenal glands of laboratory rats and mice resulting in the production of
35 corticosterone as the major glucocorticoid, instead of cortisol as in humans. Another major
36 difference involves the absence of the zona reticularis (zR) and adrenal androgen synthesis in
37 these rodents.⁸ Furthermore, potassium channels which function in the maintenance of zG cell
38 membrane potential show different gene expression profiles and patterns of immunostaining in
39 rat and human adrenals.^{9,10} Notably, the rat adrenal does not express the inwardly rectifying
40 potassium channel KCNJ5 which is frequently mutated in primary aldosteronism.⁹ Regulation of
41 *KCNJ5* gene expression and channel activity modulate the zG membrane depolarization that
42 normally initiates aldosterone production in humans.¹¹ KCNJ5 mutations allow uncontrolled zG
43 membrane depolarization and cause aldosterone excess.

44

The adrenal cortex is divided into three morphologically distinct layers (zG, zF, and zR). In the human adrenal, restricted expression of *CYP11B2* (encoding aldosterone synthase) in the zG and *CYP11B1* (encoding 11 β -hydroxylase) in the zF and zR sustains the functional zonation of aldosterone biosynthesis in the zG and cortisol in the zF. These two CYP11B enzymes, with their specific zonal distributions, are present in multiple species including mice, rats, hamsters, and guinea pigs.⁸ In contrast, others, such as pigs, cattle, sheep and dogs, express a single CYP11B enzyme that performs the final steps of both aldosterone and cortisol biosynthesis¹² and, by way of an unknown mechanism, their biosynthetic zonal specificity is maintained.^{13,14}

Large animals such as pigs are useful to model complex human diseases due to their comparable anatomy and physiology to humans.¹⁵⁻¹⁷ Such models provide an opportunity to screen for disease biomarkers and test novel therapeutic strategies.^{18,19} In this study, we evaluated the role of dietary sodium manipulation on adrenal morphology, steroidogenesis and transcriptome profiles in 6-week-old male pigs. Our objective was to identify the transcriptional response of the adrenal to RAAS activation and determine the suitability of the pig as a translational animal model for human adrenal steroidogenesis.

Methods

An expanded online methods section is available in the online supplementary file.

The authors declare that all supporting data are available within the article and its online supplementary files. mRNA-seq data are publicly available and can be accessed at <https://github.com/MedIVLMUMunich/PigAdrenalRNAseq>

69 Animal handling

70 The study used 6-week-old male German Landrace DanBred pigs on a controlled 14-day diet of
71 0.04% sodium (n= 3; low sodium group) or 0.7% sodium (n= 3; high sodium group, around 5-times
72 higher than in standard pig feed) of equivalent metabolic energy with free access to water (Table
73 S1).

74 Immunohistochemistry and immunofluorescence

75 Primary antibodies for immunohistochemistry and immunofluorescence are shown in Table S2. A
76 pig polyclonal CYP11B antibody was generated using a synthetic peptide (acetyl-
77 ⁹⁵EDVERLQKVEGLHPQR¹¹⁰C) (Figure S1) which was validated for use in Western blotting and
78 immunohistochemistry and immunofluorescence (Figure S2). CYP17A and KCNJ5 monoclonal
79 antibodies were produced as described previously.^{20,21}

80 Steroid measurements and renin assays

81 Liquid chromatography tandem mass spectrometry (LC-MS/MS) for adrenal steroid measurements
82 was performed according to Peitzsch et al.²² Renin activity was determined by LC-MS/MS
83 quantification (Attoquant Diagnostics, Vienna, Austria) as reported elsewhere.²³

84 Matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI)

85 *In situ* metabolic imaging analysis of fresh frozen adrenal sections was performed with on-tissue
86 derivatization using Girard's T reagent according to Suguira et al.²⁴ and MALDI-MSI analysis was
87 performed as reported by Sun et al.²⁵

88 Transcriptome profiling by mRNA sequencing

89 mRNA-seq transcriptome profiling was done by Eurofins Genomics (Ebersberg, Germany).

90 Statistical analysis and bioinformatics

91 IBM SPSS Statistics 26 (IBM Corp., Armonk, New York, USA) and GraphPad PRISM 8.0a (La Jolla,
92 California, USA) were used for statistical analyses. Significant differences were analyzed using a

Friedman test for repeated measures, with correction for multiple comparisons, where appropriate, and a Mann-Whitney test for independent measures. P-values less than 0.05 were considered statistically significant. Biological pathway enrichment was analyzed using Reactome (<https://reactome.org/PathwayBrowser/#/>) and Gene Ontology (<http://geneontology.org/>) databases.²⁶⁻²⁸

Results

Low dietary sodium intake and activation of the RAAS

Pigs consumed comparable amounts and ate all daily allocated food. Measurements of urinary sodium excretion levels confirmed the difference in sodium intake between the 2 groups (Figure 1A). At day 14, plasma renin activity increased 7-fold compared with day 0 reaching 116.91 pmol/L/min (9.13 ng/mL/hr \pm 3.7) under the low sodium diet. (Figure 1B).

Fourier-transform ion cyclotron resonance MSI of adrenal sections combined with on-tissue derivatization of steroids with Girard T (GirT) reagent facilitated the clear visualization of aldosterone or cortisone (which have an identical *m/z* ratio) in the zG layer of pigs on the sodium-restricted diet and low detection in the zG of pigs on the high sodium diet (Figure 1C). Because co-expression of CYP17A (which is not expressed in the zG) with CYP11B is required for cortisol and cortisone synthesis, the signal in the zG layer indicates increased synthesis of aldosterone in the zG under dietary sodium restriction.

Blood plasma steroid concentrations measured by LC-MS/MS are shown in Table S3 and are also represented in a heat map (Figure 1D) to illustrate the progressive time-related increase of plasma aldosterone levels in pigs on the sodium-restricted diet only. This corresponded to a 43.2-fold

117 increase in plasma aldosterone concentrations (day 14 *versus* day 0, $p = 0.011$) to reach 3.20
118 nmol/L (1.153 ± 0.583 ng/mL) at day 14 (Table S3). No significant differences in concentrations of
119 steroids other than aldosterone were observed. The plasma 18-hydroxycorticosterone
120 concentration progressively increased on the low sodium diet to 48.66 nmol/L (17.638 ± 11.185
121 ng/mL) at day 14 compared with 3.92 nmol/L (1.421 ± 0.082 ng/mL) at day 0, but the difference
122 was not significant. The hybrid steroids 18-hydroxycortisol and 18-oxocortisol were identified in all
123 pig blood plasma samples. In the plasma samples measured, both 18-hydroxycortisol and 18-
124 oxocortisol displayed maximum levels in the sodium restricted pigs at day 14 (18-hydroxycortisol,
125 5.79 nmol/L [2.190 ± 1.178 ng/mL]; 18-oxocortisol, 0.27 nmol/L [0.102 ± 0.046 ng/mL]).

126

127 Morphological changes in adrenal cortex in response to dietary sodium restriction

128 Pig adrenals showed strong immunostaining of VSNL1 and KCNJ5 in the zG layer (Figure 2A and B).
129 VSNL1 immunostaining highlighted an increased thickness of the zG layer from $6.5\% \pm 0.21$ of the
130 total adrenal cortex on the high sodium diet, to $13.8\% \pm 0.17$ ($p < 0.001$) on the low sodium diet.
131 Similar results were observed with measurements from KCNJ5 immunostaining (Figure 2B).

132

133 CYP11B immunohistochemistry showed positive staining throughout the adrenal cortex with more
134 intense immunostaining throughout the thickened zG layer. *CYP11B* gene expression displayed a
135 2.8 ± 0.3 -fold increase ($p = 0.045$) in the zG under sodium restriction with no change observed in
136 the zF (Figure 2C). Increased zG cell proliferation was demonstrated under sodium restriction with
137 a 2.9 ± 0.26 -fold increase ($p = 0.028$) in Ki-67 immunoreactive cells relative to pigs on a high sodium
138 diet (Figure 2D). CYP11B-CYP17A double immunofluorescence delineated the zG layer more
139 clearly, as CYP17A expression and thus immunostaining is restricted to the zF and zR, and

140 confirmed increased intensity of CYP11B immunostaining in the zG but not in the zF under the low
141 *versus* high sodium diets (Figure 3).

142

143 mRNA-seq transcriptome analysis of the zG under a low and high sodium diet

144 An overview of the mRNA-seq analysis is shown in Figure S3. Comparison of the transcriptomes of
145 the zG and zF demonstrated a higher number of transcripts with significantly altered expression
146 levels in the zG than the zF (Figure S3, Figure 4A-C). These included 1,172 significantly altered
147 annotated genes in the zG of the low *versus* high sodium groups comprising 768 upregulated and
148 404 downregulated genes:

149 [https://github.com/MedIVLMUMunich/PigAdrenalRNAseq/raw/master/1\)zG_All%20annotated%20](https://github.com/MedIVLMUMunich/PigAdrenalRNAseq/raw/master/1)zG_All%20annotated%20DEGs.xlsx)
150 [ODEGs.xlsx](https://github.com/MedIVLMUMunich/PigAdrenalRNAseq/raw/master/1)zG_All%20annotated%20DEGs.xlsx). In the zF, 280 significantly altered annotated genes were identified with 172
151 upregulated and 108 downregulated:

152 [https://github.com/MedIVLMUMunich/PigAdrenalRNAseq/raw/master/2\)zF_All%20annotated%20](https://github.com/MedIVLMUMunich/PigAdrenalRNAseq/raw/master/2)zF_All%20annotated%20DEGs.xlsx)
153 [ODEGs.xlsx](https://github.com/MedIVLMUMunich/PigAdrenalRNAseq/raw/master/2)zF_All%20annotated%20DEGs.xlsx).

154

155 Significantly altered transcripts which were common to the zG and the zF under low *versus* high
156 dietary sodium comprised 89 upregulated and 30 downregulated genes. In addition, 6 transcripts
157 were upregulated in the zG but downregulated in the zF (*FHIT*, *ssc-mir-202*, *WWOX*, *GTDC1*,
158 *ZNF592* and *SLC2A9*) and 1 transcript was downregulated in the zG and upregulated in the zF
159 (*HEATR3*) in response to salt restriction (Figure 4C).

160

161 A heat map representation of differentially expressed genes (DEGs) (defined as a log₂-fold change
162 ≥ 2) under low *versus* high sodium intake in the zG (59 genes) and the zF (21 genes) is shown in
163 Figure 4D-E. The top 20 upregulated and downregulated genes in the zG (low *versus* high sodium

164 diet) with gene loci, expression levels and respective functions are shown in Table S4 and in the zF
165 in Table S5. Several DEGs were identified with a described role in aldosterone production or
166 adrenal function. These included genes encoding the transcription factors FOSB, FOS, VDR and
167 NR5A1 (also called SF-1), RGS4 (regulator of G protein signaling 4), the calcium-binding proteins
168 VSNL1 (visinin-like 1) and SMOC (secreted modular calcium-binding protein 1), STARD4 (StAR-
169 related lipid transfer protein 4), the cytochrome P450 enzymes CYP27A1, CYP21A2 and CYPB1, and
170 VAV2 (Vav Guanine Nucleotide Exchange Factor 2) (Table).²⁹⁻⁴³

171

172 Enriched biological pathways in the zG in response to dietary sodium restriction

173 Biological pathway analysis of significantly DEGs in the pig adrenal zG transcriptomes (low *versus*
174 high sodium diet) identified enriched pathways related to steroid metabolism ($p= 5.17\text{e-}2$; number
175 of DEGs= 31), cholesterol biosynthesis ($p= 2.51\text{e-}4$; DEGs= 12) and regulation of cholesterol
176 biosynthesis by SREBP (sterol regulatory-element binding protein; $p= 9.79\text{e-}6$; DEGs= 12), the cell
177 cycle ($p= 9.82\text{e-}1$; DEGs= 40) and potassium channels ($p= 5.18\text{e-}1$; DEGs= 9) (Table S6). The DEGs
178 in the steroid metabolism pathway included *VDR* (vitamin D receptor), the top DEG related to this
179 pathway, and *STARD4* (StAR related lipid transfer domain containing 4). Seven DEGs were
180 common to the regulation of cholesterol biosynthesis by SREBP and the cholesterol biosynthesis
181 pathways (*HMGCS1*, *HMGCR*, *FDFT1*, *PMVK*, *MVK*, *SQLE* and *MVD*). DEGs associated with the cell
182 cycle included *AGTR2* (angiotensin II receptor type 2) and *PRKAR2B* (cAMP-dependent protein
183 kinase type II-beta regulatory subunit). *KCNJ5* was the DEG in the potassium channel pathway with
184 the highest expression level in the zG (Table S6).

185

186

187

188 Discussion

189 We demonstrate the effect of RAAS activation by dietary sodium restriction on adrenal
190 morphology, steroidogenesis and transcriptome profiles in pigs as a translational animal model for
191 humans. In pigs, as described previously in rats,^{4,5} RAAS activation induced by dietary sodium
192 restriction caused zG expansion, increased zG expression of aldosterone synthase (called CYP11B
193 in pigs and CYP11B2 in rats and humans) at both transcript and protein levels and an increased
194 production of aldosterone from the zG. After 14 days, pig plasma aldosterone concentrations were
195 comparable to those reported in Yanomami Indians (3.20 nmol/L *versus* 2.38 nmol/L,
196 respectively), a population noted for their low salt consumption⁴⁴, and 20-fold higher than in a
197 group of 525 adult humans from Europe.⁴⁵

198

199 We show strong immunostaining of KCNJ5 in the zG of the pig adrenal as in humans²¹ and *KCNJ5*
200 transcripts were detected in the zG but not the zF. KCNJ5 is a potassium inwardly rectifying
201 channel that contributes to normal membrane polarization and may contribute to the mechanism
202 of aldosterone synthesis in response to angiotensin II stimulation.¹¹ Germline and somatic *KCNJ5*
203 mutations that cause membrane depolarization of the zG cell have been identified as the main
204 known molecular variants causing primary aldosteronism in humans.⁴⁶ However, there are
205 redundant mechanisms for the maintenance of membrane potential and knocking down the
206 expression of KCNJ5 in human adrenal cortical carcinoma cells did not alter basal aldosterone
207 synthesis nor abrogate its stimulation by angiotensin II.¹¹ *KCNJ5* transcripts were undetectable in
208 laser-captured samples of rat adrenal zG and specific KCNJ5 immunostaining was undetectable
209 indicating a difference between rats and humans in the regulation of zG membrane potential and
210 aldosterone production.⁹

211

212 We used mRNA-seq analysis to gain further insight into transcriptome changes of zG cells
213 associated with increased aldosterone synthesis in the pig as a model for the regulation of human
214 aldosterone production. RNA-seq analysis offers the possibility to accurately quantify expression
215 of genes from very low to high levels and allowed the identification of a large number of genes in
216 the zG transcriptome (768 and 404 up- and down-regulated annotated genes) which are regulated
217 by chronic RAAS activation compared with the rat using microarray analysis (201 and 68 up- and
218 down-regulated genes, low *versus* high sodium diet).⁵ In the latter case, the rats were fed a
219 sodium-controlled diet for 3 days, compared with the 14-days in the current study, and the high
220 dietary sodium levels used in the rat study were almost 10-times greater than in the present
221 study, with relatively mild high sodium conditions, which may also account for this difference.

222
223 We report several upregulated genes in the zG in response to sodium restriction with a previously
224 reported role in aldosterone production. *VDR* (encoding the vitamin D receptor) was the top DEG
225 in the zG with an annotated function related to steroid metabolism which was expressed at a low
226 level in the zG and undetected in the zF. *VDR* gene expression was upregulated by angiotensin II
227 stimulation of a human adrenocortical cell line and *VDR* overexpression caused an increase in
228 aldosterone secretion under basal and angiotensin II stimulated conditions.²⁹ The *VDR* gene is
229 upregulated in aldosterone-producing adenomas, a major cause of primary aldosteronism,
230 compared with normal adrenals⁴⁷ and *VDR* gene expression is positively correlated with *CYP11B2*
231 mRNA levels consistent with a role in pathophysiological aldosterone production.⁴⁸ Other
232 upregulated genes in the pig zG with a previously reported role in aldosterone secretion include
233 *RGS4* (regulator of G protein signaling 4) which is upregulated in the rat adrenal by a low sodium
234 diet and angiotensin II infusion^{6,30} and *VSNL1* encoding the calcium sensor protein VSNL1 which

235 regulates basal and angiotensin II-stimulated CYP11B2 gene expression in human adrenocortical
236 cells *in vitro*.³⁴
237
238 Transcriptome alterations were also identified in the zF transcriptome by stimulation of the RAAS.
239 This is consistent with the report of a more than 2-fold significant increase in cortisol secretion, in
240 addition to an increase in aldosterone secretion, by angiotensin II stimulation of human
241 adrenocortical cells *in vitro*.⁴⁹ Furthermore, an analysis of transcription regulatory genes
242 modulated by angiotensin II in cultured human adrenocortical cells identified several genes which
243 significantly activate an 11 β -hydroxylase reporter gene, with a relatively much smaller effect on
244 the expression of an aldosterone synthase reporter plasmid.⁵⁰ These genes included members of
245 the *FOS* (fos proto-oncogene) gene family, *FOS* and *FOSB*, which encode leucine zipper proteins
246 which dimerize with proteins of the *JUN* family (Jun proto-oncogene), to form the transcription
247 factor complex AP-1 (activator protein-1). *FOS* and *FOSB* were highly upregulated in the zF by
248 sodium restriction in the present study. *FOSB* transcripts were not detected in the zG whereas *FOS*
249 was expressed in both zones with a greater transcriptional response in the zF.
250
251 The cell cycle was one of the most enriched biological pathways in the zG in sodium restricted pigs
252 reflecting the expansion of the zG layer, in agreement with a zG microarray analysis in rats.⁵
253 *AGTR2* (angiotensin II type 2 receptor) transcription showed the highest response of the DEGs
254 annotated to the cell cycle in the zG. The *AGTR2* is highly expressed in the human fetal adrenal,³¹
255 strikingly higher than in the adult,³² where it may mediate apoptosis during adrenal gland
256 development.³³ The pronounced altered expression of *AGTR2* in the zG transcriptome in response
257 to sodium restriction suggests a potential role in the control of adrenal morphology after birth.
258

259 The strengths of our study include the use of an animal species closely related to humans. We
260 used a relatively mild high sodium diet to represent a level of salt intake relevant to that of many
261 humans which is in contrast to the highly abnormal experimental conditions of 4-8% NaCl in high
262 salt diets given to rats. Moreover, unlike in rats and mice, pigs display clear, high-level KCNJ5
263 expression in the zG, and produce the hybrid steroids 18-hydroxycortisol and 18-oxocortisol which
264 display elevated levels in patients with primary aldosteronism carrying KCNJ5 mutations.^{51,52} This
265 highlights the superiority of the pig over rodents for the study of human adrenal biology and
266 indicates the potential utility of the pig to model aspects of primary aldosteronism.

267
268 A limitation of our study is the low number of animals used in each diet group, which may explain
269 why differences in aldosterone precursor steroids were not detected despite the identification of
270 differences in aldosterone production. We also used only male animals to circumvent gender-
271 related effects on steroid production⁴⁵ and thus we could not address sex-related effects of dietary
272 sodium on aldosterone production.⁵³ An additional limitation of the pig is the expression of a
273 single CYP11B enzyme in the adrenal cortex. Despite this, shared mechanisms of aldosterone
274 physiology with animals that express both CYP11B2 and CYP11B1 in their adrenals are likely
275 because many genes involved in the transcriptional response to sodium restriction were identified
276 common to pigs and rats.

277

278 **Perspectives**

279 This study presents a proof of concept for the suitability of the pig as a model of human
280 steroidogenesis and provides a rich source of transcriptome data for studies on aldosterone
281 physiology and pathophysiology and for the identification of potential novel pharmacological
282 targets to treat aldosterone excess.

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Conflicts of interest/Disclosure

None

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Novelty and Significance

What is new?

- The level of sodium intake significantly alters pig adrenal morphology, steroidogenesis and transcriptome profiles
- Under a low sodium diet, the pig adrenal displays zona glomerulosa expansion with increased expression of CYP11B
- MALDI-MSI demonstrated that aldosterone production in the zona glomerulosa increased in response to sodium restriction
- Plasma aldosterone concentrations and plasma renin activity were increased in pigs fed a low sodium diet for 14-days
- The KCNJ5 potassium channel is highly expressed in the pig zona glomerulosa and absent in the zona fasciculata
- Use of the pig adrenal as a surrogate to study adrenal steroidogenesis offers advantages over that of the rat or mouse

What is relevant?

- Pig adrenal transcriptome profiling by mRNA seq analysis identifies genes responsive to activation of the renin-angiotensin aldosterone system
- An abundance of genes in the zona glomerulosa and the zona fasciculata respond to dietary sodium restriction
- In some respects, pig adrenal steroidogenesis is more like that of the human than the adrenals of rats and mice.

Summary

The pig is an appropriate model to study the regulation of aldosterone secretion and will be useful to discover and assess novel pharmacological targets of aldosterone excess.

Figure Legends

Figure 1. Activation of the pig renin-angiotensin-aldosterone system by dietary sodium restriction

Fractional excretion of sodium measurements confirmed the higher sodium intake at 7 and 14 days of pigs on the high compared with the low sodium diet (**Panel A**). Plasma renin activity showed a 7-fold increase at day 14 compared with day 0 (**Panel B**). MALDI-MSI demonstrated the increased aldosterone or cortisone production in the zG of the low sodium group. The absence of CYP17A (required for cortisol synthesis) in the zG indicates that the increased intensity of the aldosterone or cortisone signal in the zG is aldosterone. The zG is delineated with white broken lines (**Panel C**). Multiple adrenal steroids were measured by LC-MS/MS showing the progressive increase of aldosterone production up to day 14 in pigs under sodium restriction. Data for individual pigs is normalized to the average plasma steroid concentration at day 0 for high and low sodium groups and the intensity scale indicates fold change over baseline (**Panel D**).

FENa, fractional excretion of sodium; GirT, Girard's Reagent T; H&E, hematoxylin and eosin; 18OH-cortisol, 18-hydroxycortisol; 18OH-corticosterone, 18-hydroxycorticosterone; 17OH-progesterone, 17-hydroxyprogesterone; PRA, plasma renin activity. Bars represent mean \pm SEM (n=3 for each group), a Friedman non-parametric test for repeated measures was used to detect paired differences. Circles, high sodium; triangles, low sodium diet. *p<0.05. Panel C, scale bar = 1mm and 200 μ m in zoomed images as shown.

Figure 2. Morphological and functional expansion of the pig adrenal zona glomerulosa by dietary sodium restriction

Immunostaining of VSNL1 and KCNJ5 highlighted the increase in the zG in the pigs fed a 14-day low sodium diet. The thickness of the zG layer is shown as a percentage of the total thickness of the adrenal cortex. The average of 6 measurements for each adrenal is shown (**Panel A and B**). CYP11B immunostaining shows the expansion of the zG layer is complemented by an increase in aldosterone synthase-positive cells in pigs on a low sodium diet (**Panel C**) and immunostaining for Ki67, a marker of cell proliferation, shows a significant increase in Ki67-positive cells in the zG layer on a low *versus* high sodium diet (**Panel D**). Bars represent mean \pm SEM (n=3 per group), a Mann-Whitney test was used to detect differences. Circles, high sodium; triangles, low sodium diet. *p<0.05, ***p<0.001.

Figure 3. CYP11B-CYP17A double immunofluorescence staining

In the adrenal, CYP17A is exclusively expressed in the zF and the zR and CYP11B-CYP17A double immunostaining allows the clear visualization of the zG layer showing the increased thickness and increased CYP11B immunostaining in the zG compared with the zF in pigs on the low compared with the high sodium diet. The zG is delineated with white broken lines. Scale bar= 50 μ m

Figure 4. Transcriptome alterations in the zona glomerulosa and the zona fasciculata under low *versus* high sodium diets.

Volcano plots indicate transcripts with significantly altered levels (red dots) by dietary sodium manipulation in the zG (**Panel A**) and zF (**Panel B**). The numbers of significantly altered transcripts in the zG and zF (low *versus* high dietary sodium) are indicated in the Venn diagram (**Panel C**). There were 6 transcripts upregulated in response to sodium restriction in the zG which were

downregulated in the zF and one transcript which was downregulated in the zG but upregulated in the zF (low *versus* high sodium). The heat maps represent the up- and down-regulated genes (defined as log₂-fold change>2) in the zG (**Panel D**) and zF (**Panel E**) of each pig (n= 3 in each of the high and low sodium diet groups). The gene names are indicated on the left of each heat map and log₂-fold change on the right. FC, log₂-fold change; zG, zona glomerulosa; zF, zona fasciculata

Figure 1.

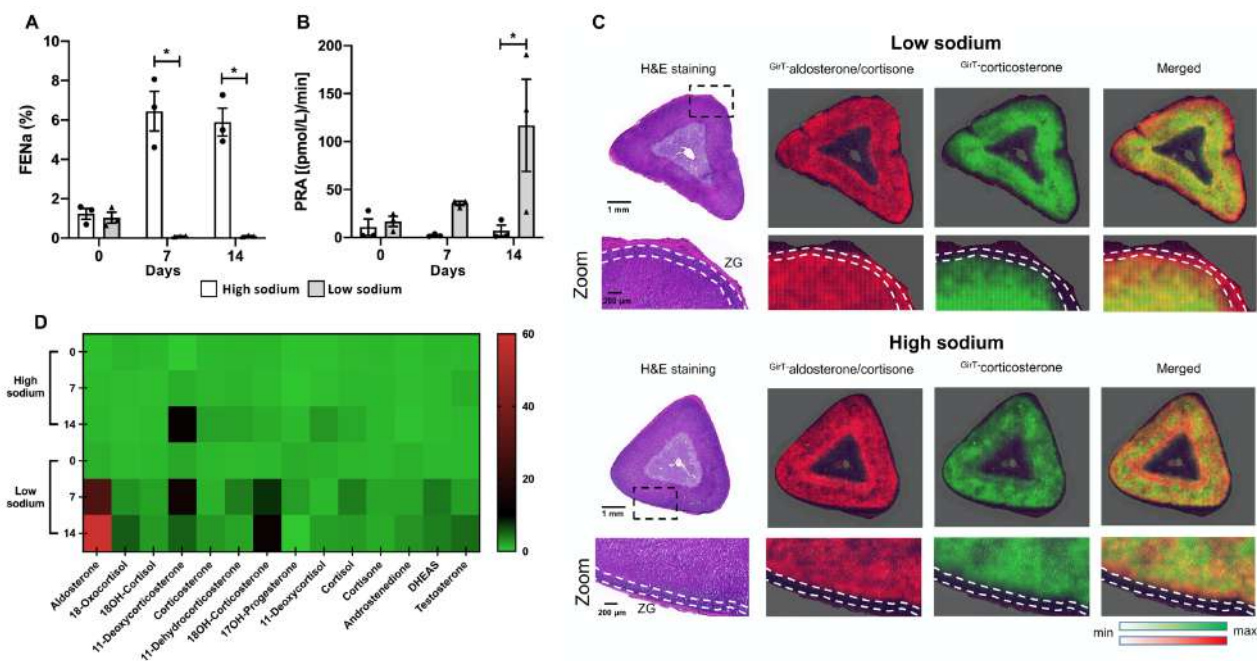


Figure 2.

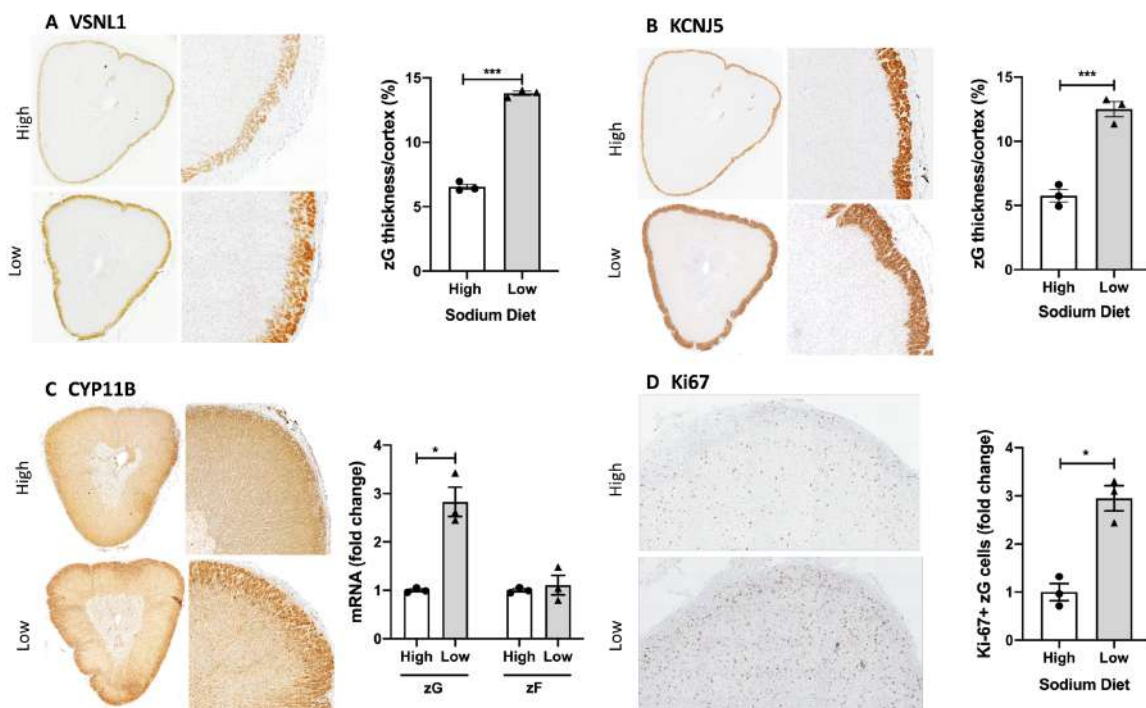
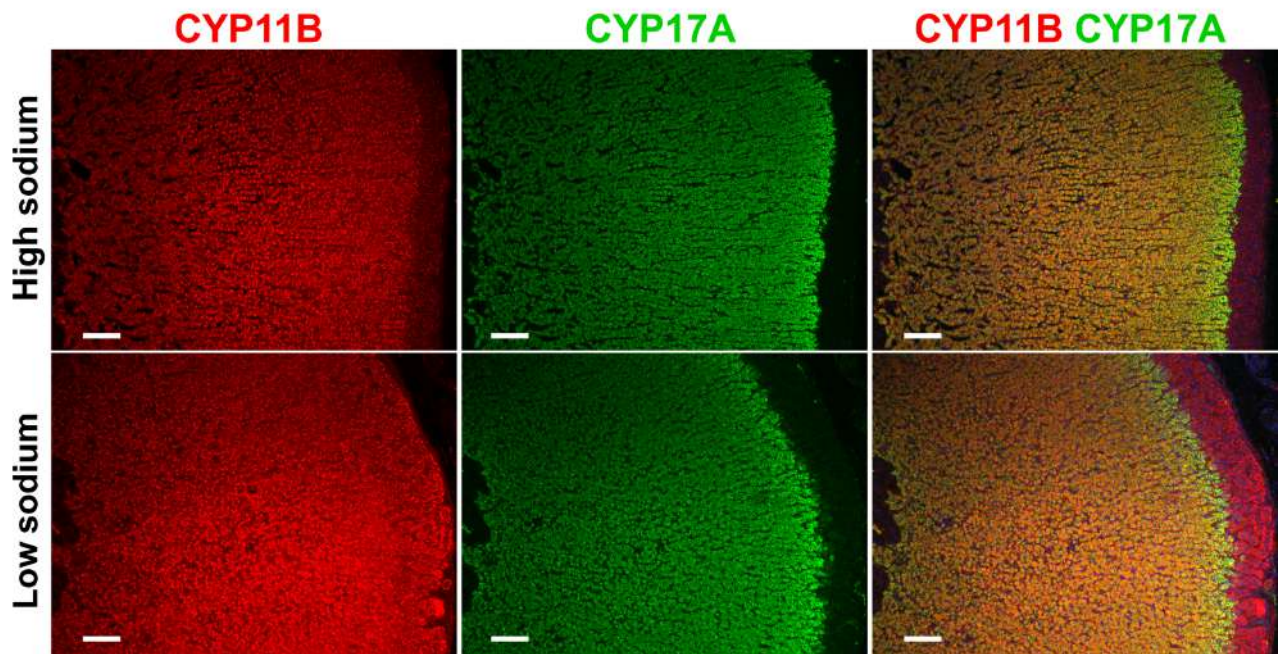
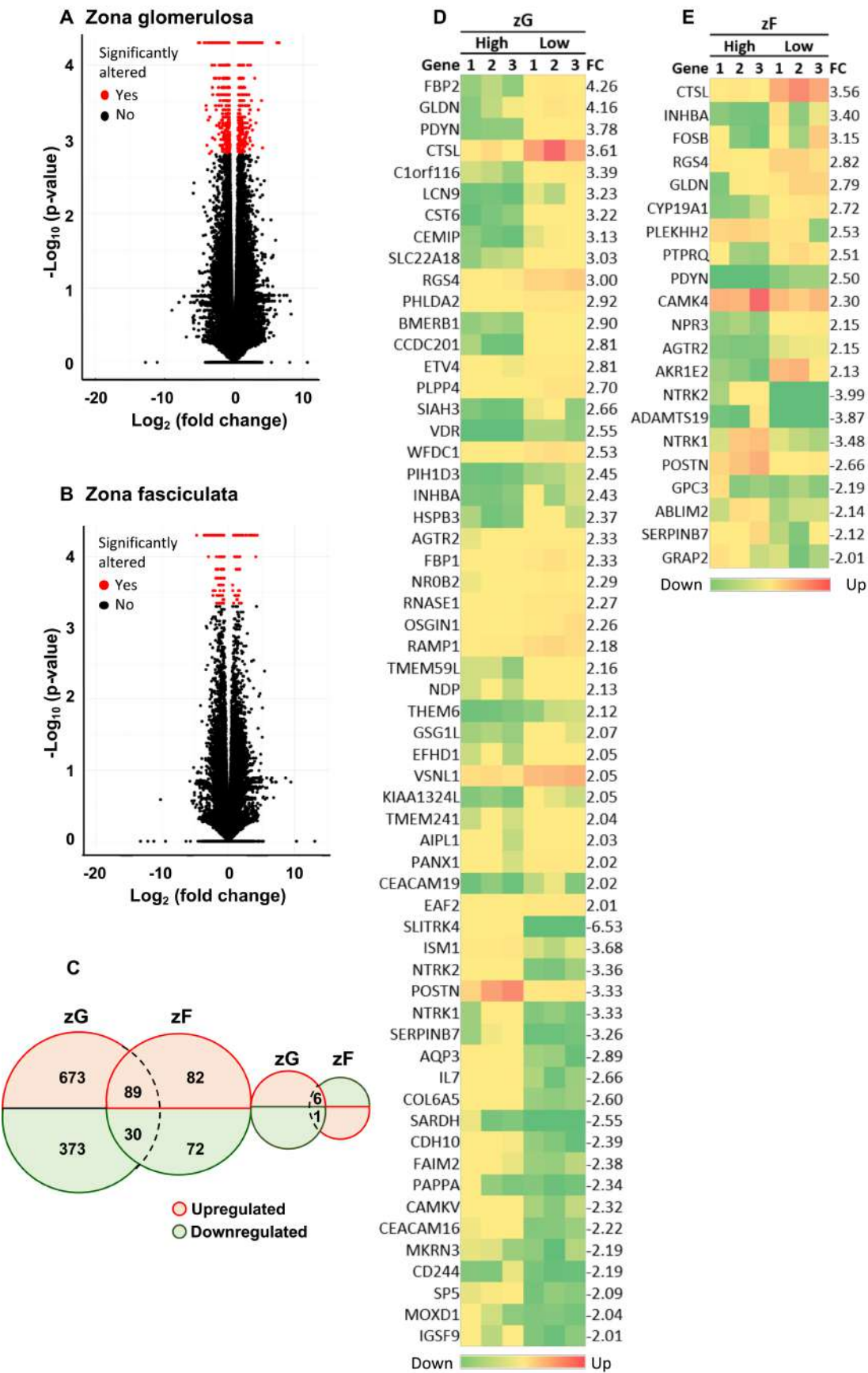


Figure 3.



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Figure 4.



Gene	zG_FPKM		Log ₂ _fc LS vs. HS	P value	zF_FPKM		Log ₂ _fc LS vs. HS	P value	REF
	LS	HS			LS	HS			
FOSB	n.d.	n.d.	-	-	8.25	0.93	3.15	0.00005	29
RGS4	174.01	21.70	3.00	0.00005	21.96	3.10	2.82	0.00005	6, 30
VDR	1.63	0.28	2.55	0.00005	n.d.	n.d.	-	-	29
AGTR2	19.85	3.93	2.33	0.00005	1.94	0.44	2.15	0.00005	31-33
VSNL1	346.14	83.42	2.05	0.00005	34.53	8.78	1.98	0.00005	34
SMOC2	4.31	14.45	-1.75	0.00005	3.11	6.91	-1.15	0.00005	30
STARD4	16.91	8.72	0.95	0.0001	12.66	3.76	1.75	0.00005	35
FOS	55.93	28.42	0.98	0.00005	164.14	55.98	1.55	0.00005	36
CYP27A1	14.10	32.12	-1.19	0.00005	n.d.	n.d.	-	-	37
NR5A1	229.67	118.83	0.95	0.00005	n.d.	n.d.	-	-	38
VAV2	27.20	14.46	0.91	0.00005	n.d.	n.d.	-	-	39
KCNJ5	221.11	118.62	0.90	0.00005	n.d.	n.d.	-	-	40
CYP21A2	5786.23	3275.67	0.82	0.00025	n.d.	n.d.	-	-	41
CEBPB	74.41	42.09	0.82	0.00005	n.d.	n.d.	-	-	42
CYP11B1	23.04	38.24	-0.73	0.00025	n.d.	n.d.	-	-	43

Table. Top differentially expressed genes with a described functional role in the adrenal

The genes with the highest level of differential expression with a described functional role in the adrenal are shown. The table indicates the expression levels and log2-fold changes under a low versus high sodium diet in either the zG or zF.

The full list of annotated DEGs in the zG can be downloaded at:

[https://github.com/MedIVLMUMunich/PigAdrenalRNAseq/raw/master/1\)zG_All%20annotated%20DEGs.xlsx](https://github.com/MedIVLMUMunich/PigAdrenalRNAseq/raw/master/1)zG_All%20annotated%20DEGs.xlsx)

The full list of annotated DEGs in the zF can be downloaded at:

[https://github.com/MedIVLMUMunich/PigAdrenalRNAseq/raw/master/2\)zF_All%20annotated%20DEGs.xlsx](https://github.com/MedIVLMUMunich/PigAdrenalRNAseq/raw/master/2)zF_All%20annotated%20DEGs.xlsx)

Fc, fold change; FPKM, fragments per kilobase of transcript, per million mapped reads; n.d., not detected; REF, reference